

## ENZYMES OF ECDYSTEROID 3-EPIMERIZATION IN MIDGUT CYTOSOL OF *MANDUCA SEXTA*: pH OPTIMA COSUBSTRATE KINETICS, AND SODIUM CHLORIDE EFFECT\*

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**Abstract**—Five enzyme activities in midgut cytosol of *Manduca sexta* last instar larvae are potentially involved in the interconversion of 3 $\beta$ -hydroxyecdysteroids, 3-oxoecdysteroids, and 3 $\alpha$ -hydroxyecdysteroids. A Sephadex G-25-filtered high-speed supernatant was used to determine some of the characteristics of the corresponding enzymes. The pH optima of ecdysone oxidase and NADH-dependent 3-oxoecdysteroid 3 $\alpha$ -reductase were 7.5, the pH of the midgut cytosol was 7.9. The apparent kinetic parameters for the NADH-dependent 3 $\alpha$ -reductase were  $K_m$  (for NADH) =  $80.8 \pm 10.8 \mu\text{M}$  and  $V_{\max} = 0.58 \pm 0.30 \text{ nmol/min/mg protein}$ , and for the NADPH-dependent 3-oxoecdysteroid 3 $\beta$ -reductase,  $K_m$  (for NADPH) =  $19.3 \pm 2.5 \mu\text{M}$  and  $V_{\max} = 4.39 \pm 0.40 \text{ nmol/min/mg protein}$ .  $\text{NAD}^+$  and  $\text{NADP}^+$  inhibited the enzymatic 3-oxoecdysteroid reductions, but the reactions were not reversible (i.e. no conversion of ecdysone or 3-epiecdysone to 3-dehydroecdysone). Sodium chloride (0.2 M) inhibited the 3 $\alpha$ -reductase activity with NADH and strongly increased the 3 $\alpha$ -reductase activity with NADPH.

**Key Word Index:** Ecdysone oxidase; 3-oxoecdysteroid 3-reductase; 3-epiecdysone; 3-dehydroecdysone; 3 $\alpha$ -hydroxyecdysteroid; 3 $\beta$ -hydroxyecdysteroid; *Manduca sexta*; midgut; cytosol; NADH; NADPH; sodium chloride

### INTRODUCTION

3 $\alpha$ -Hydroxyecdysteroids (e.g. 3-epiecdysone, 3-epi-20-hydroxyecdysone) are found in many insect species and are presumed to be inactivation products of the biologically much more active 3 $\beta$ -hydroxyecdysteroids (Rees, 1989; Lafont and Connat, 1989; Thompson *et al.*, 1990). The first reported *in vitro* conversion of ecdysone (E) to 3-epiecdysone (E') (= 3-epimerization) was accomplished with a soluble enzyme preparation from *Manduca sexta* L. midgut and was thought to be a two-step reaction, consisting of an oxygen-dependent oxidation of E to 3-dehydroecdysone (3D), and a stereospecific reduction of 3D to E' that required NADH or NADPH as cosubstrate (Nigg *et al.*, 1974; Mayer *et al.*, 1979) (Fig. 1). In recent years this reaction mechanism has been verified

in studies with enzyme preparations from (mid)guts of *Pieris brassicae* (Blais and Lafont, 1984), *Spodoptera littoralis* (Milner and Rees, 1985), and *Manduca sexta* (Weirich, 1989; Weirich *et al.*, 1990a). These studies have also demonstrated the existence of another reductase, catalyzing the reconversion of the 3-oxo intermediate to the 3 $\beta$ -hydroxyecdysteroid (3-oxoecdysteroid 3 $\beta$ -reductase).

We recently reported the potential interrelationships of the *M. sexta* ecdysone oxidase and 3-oxoecdysteroid 3-reductases and the apparent kinetic parameters obtained by varying the concentrations of the ecdysteroid substrates (Weirich *et al.*, 1990a). In the present communication we report the pH optima, the apparent kinetic parameters obtained by varying the concentrations of the nicotinamide adenine dinucleotide cosubstrates, and the effects of oxidized dinucleotides ( $\text{NAD}^+$ ,  $\text{NADP}^+$ ) and of sodium chloride on the *M. sexta* enzymes.

\*Mention of a company name or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

**Abbreviations used:** E, ecdysone; E', 3-epiecdysone; 3D, 3-dehydroecdysone; G-25 sup, combined protein fractions of 80,000 g supernatant of *M. sexta* midgut homogenate, obtained by gel filtration on Sephadex G-25; 3 $\alpha$ -reductase (NADH), 3 $\alpha$ -hydroxyecdysteroid:NAD $^+$  oxidoreductase; 3 $\alpha$ -reductase (NADPH), 3 $\alpha$ -hydroxyecdysteroid:NADP $^+$  oxidoreductase; 3 $\beta$ -reductase (NADH), 3 $\beta$ -hydroxyecdysteroid:NAD $^+$  oxidoreductase; 3 $\beta$ -reductase (NADPH), 3 $\beta$ -hydroxyecdysteroid:NADP $^+$  oxidoreductase; Glc-6-P, glucose 6-phosphate.

### MATERIALS AND METHODS

#### Chemicals

Ecdysone was obtained from Simes Pharmaceuticals (Milan, Italy); E' and 3D were synthesized according to published procedures (Spindler *et al.*, 1977).

$\text{NAD}^+$ , grade V-C; NADH, grade III;  $\text{NADP}^+$ , Sigma grade; NADPH, type X; and *Leuconostoc mesenteroides* Glc-6-P dehydrogenase were obtained from Sigma Chemical Co. (St Louis, Mo.) and Sephadex G-25 (fine), from Pharmacia LKB Biotechnology (Piscataway, N.J.).

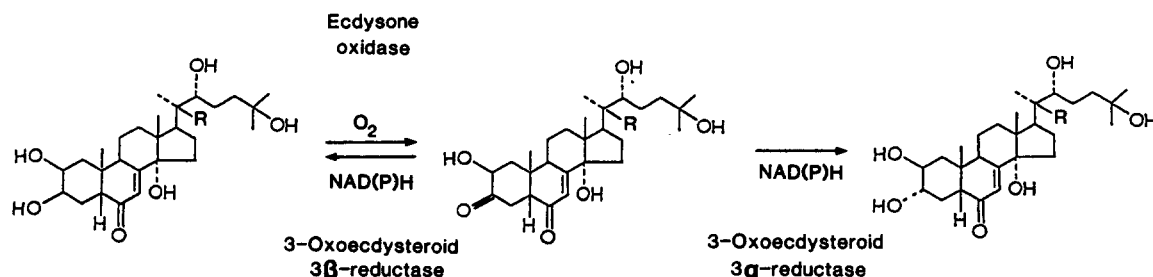


Fig. 1. Enzymatic interconversions of  $3\beta$ -hydroxyecdysteroids, 3-oxoecdysteroids, and  $3\alpha$ -hydroxyecdysteroids. R = H, ecdysone series; R = OH, 20-hydroxyecdysone series.

### Enzyme preparation

Tobacco hornworms (*M. sexta*) were reared to the late fifth instar on artificial diet (Hoffmann *et al.*, 1966). Midguts of "wandering" larvae were dissected and homogenized, and the 80,000 *g* ( $3.6 \times 10^{10}$  rad<sup>2</sup> s<sup>-1</sup>) supernatant was prepared as described previously (Weirich and Adams, 1984; Thompson *et al.*, 1985). For the determination of the cytosol pH the Tris-sucrose-EDTA isolation buffer was substituted by 0.15 M potassium chloride solution.

The supernatant was fractionated on Sephadex G-25 (Weirich *et al.*, 1986), equilibrated with 12.5 mM Tris-HCl buffer, pH 7.5, containing 1.0 mM EDTA (buffer A). The combined protein fractions (G-25 sup) were used for the incubations.

Protein concentrations were determined according to Lowry *et al.* (1951) with bovine serum albumin as standard.

### Incubations

**Ecdysone oxidase.** Ecdysone (25–50  $\mu$ M) and G-25 sup (2.1 mg protein) were incubated for 120 min at 30°C (Dubnoff metabolic incubator, 90–100 oscillations/min), in 1.0 ml of 30 mM potassium phosphate-10 mM Tris-HCl buffer, pH 7.5, containing 1.0 mM EDTA (buffer B). After a 5–10 min equilibration period, the reactions were started by the addition of E, dissolved in 5  $\mu$ l of methanol. The incubations were stopped by the addition of 4.0 ml methanol.

**3-Oxoecdysteroid 3-reductases.** Incubation mixtures contained 3D (12.5–40  $\mu$ M) and G-25 sup in 0.5–2.0 ml of buffer B or buffer C (same composition as buffer B, but pH 6.5). The concentrations of G-25 sup proteins in the incubation mixtures were 0.19–1.70 mg/ml for incubations containing NADH as cosubstrate, 0.07–0.13 mg/ml for incubations containing NADPH as cosubstrate. Except for the kinetic experiments, NADH or NADPH were added to the incubation mixtures to a final concentration of 0.6 mM.

The reaction mixtures were equilibrated in a Dubnoff metabolic incubator for 5–10 min (30 min for anaerobic incubations) at 30°C and 90–100 oscillations/min. The reactions were started by addition of 3D, dissolved in 50–100  $\mu$ l of buffer A (for anaerobic incubations, injected through the venting tube). Incubation times were 10–20 min, and the incubations were stopped by addition of 4.0 ml methanol.

**Anaerobic incubations.** Ecdysone oxidase in the G-25 sup can reoxidize ecdysone formed by  $3\beta$ -reductase in the incubation mixtures. As a result, the activity of the  $3\beta$ -reductase will be slightly underestimated. Therefore, incubations of G-25 sup, 3D, and NADPH (generating substantial amounts of E) were carried out anaerobically. The small yields of E from NADH-containing incubations were not significantly affected by reoxidation.

Vials (20 ml) containing the incubation mixtures were covered with rubber stoppers into which one gassing tube and one venting tube had been inserted. The incubation mixtures were purged with water-saturated nitrogen at approx. 100 ml/min.

**pH optima.** For the determination of the pH optima the incubation mixtures were buffered with sodium citrate/phosphate (for pH 5–7), sodium phosphate (for pH 7–8), or glycine/NaOH/sodium phosphate (for pH 8–10). All buffers were made to an ionic strength of 0.20 and contained 1.0 mM EDTA. No conversions of E or 3D resulted from control incubations without added enzyme preparation in any of these buffers.

**Kinetic experiments.** NADH or NADPH concentrations were 5–336  $\mu$ M and were obtained by addition of the appropriate amounts of NAD<sup>+</sup> and NADP<sup>+</sup>, and a regenerating system consisting of 2.5 units of *L. mesenteroides* Glc-6-P dehydrogenase/ml, and 6.0 mM Glc-6-P. *L. mesenteroides* Glc-6-P dehydrogenase reacts with NAD<sup>+</sup> and NADP<sup>+</sup> at different rates, and the amount of enzyme added to the incubations was adjusted accordingly (i.e. 2.5 "NAD<sup>+</sup> units" for incubations requiring NADH as cosubstrate, 2.5 "NADP<sup>+</sup> units" for incubations requiring NADPH as cosubstrate). This regenerating system provided constant NADH and NADPH concentrations (within  $\pm 5\%$ ) during the incubations (monitored by u.v. absorption at 340 nm).

In the range of G-25 sup protein concentrations used, the reactions were linear for the duration of the incubations (10 min), and the reaction rates were proportional to the amounts of protein added.

The reciprocals of substrate concentrations and velocities were subjected to linear regression analysis and a 2-tailed *t*-test. Only sets of data that resulted in a confidence limit of  $\geq 99\%$  were used for the calculation of the apparent kinetic parameters.

### Extraction, purification and HPLC analysis

The reaction products were extracted, purified and analyzed by reversed-phase HPLC as previously reported (Weirich *et al.*, 1990a).

## RESULTS

In the interconversion of  $3\alpha$ -hydroxyecdysteroids, 3-oxoecdysteroids, and  $3\beta$ -hydroxyecdysteroids five different enzyme activities can be distinguished (Fig. 1): ecdysone oxidase, NADH-dependent 3-oxoecdysteroid  $3\alpha$ -reductase, NADH-dependent 3-oxoecdysteroid  $3\beta$ -reductase, NADPH-dependent 3-oxoecdysteroid  $3\alpha$ -reductase, and NADPH-dependent 3-oxoecdysteroid  $3\beta$ -reductase (Weirich *et al.*, 1990a). It is not clear at present whether the reductions are catalyzed by one  $3\alpha$ -reductase and one  $3\beta$ -reductase, each displaying different kinetic parameters with each of the two nicotinamide adenine dinucleotide cosubstrates, or by four different cosubstrate-specific reductases. However, for the sake of simplicity they will be referred to as four individual enzyme activities with the cosubstrate shown in parentheses.

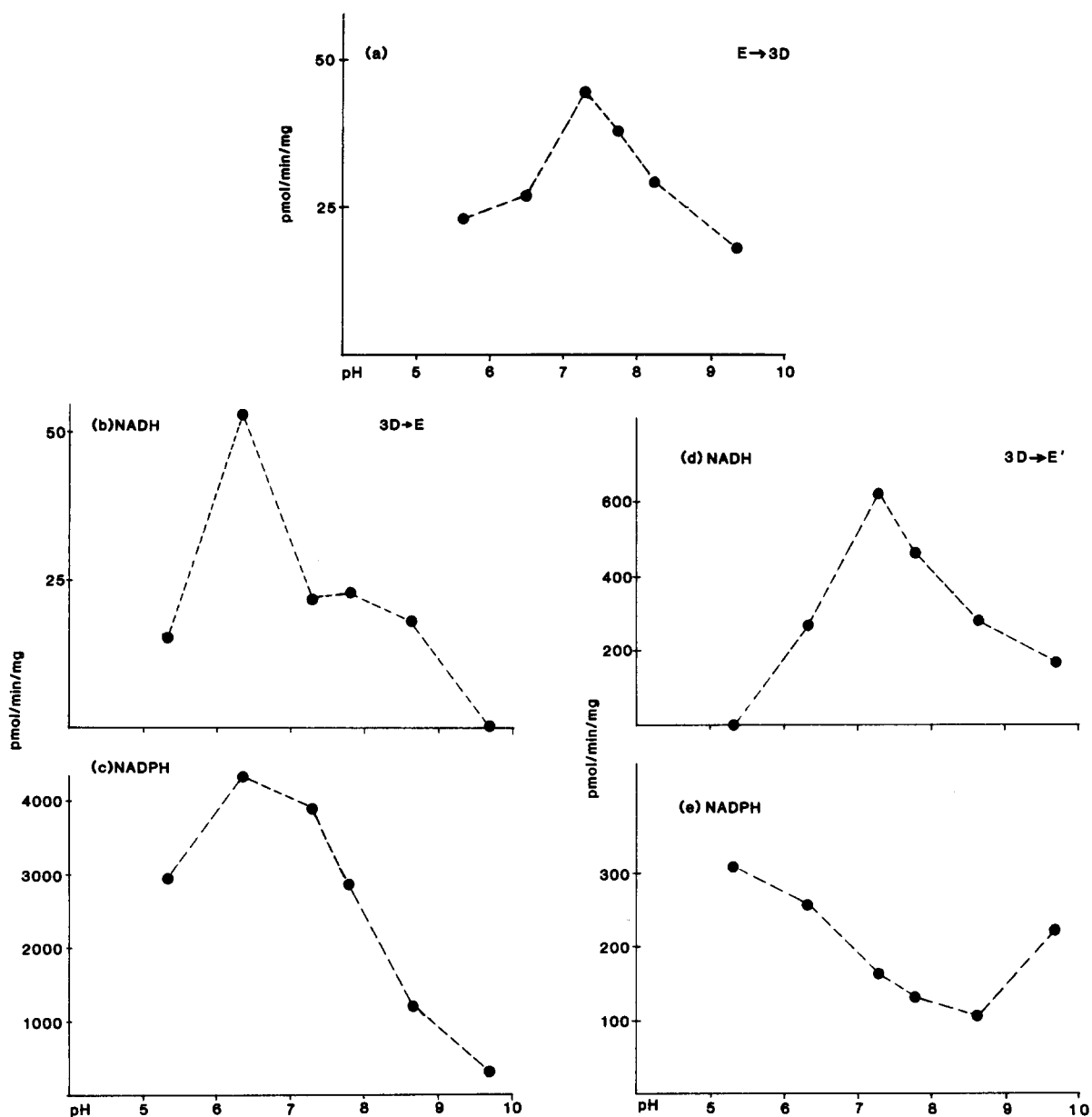


Fig. 2. Effect of pH on ecdysone oxidase (a),  $3\beta$ -reductase (NADH) (b),  $3\beta$ -reductase (NADPH) (c),  $3\alpha$ -reductase (NADH) (d), and  $3\alpha$ -reductase (NADPH) (e). Incubation mixtures contained  $50 \mu\text{M}$  E (a) or  $40 \mu\text{M}$  3D (b-e), and G-25 sup [0.12 (c, e), 0.49 (b, d), or 2.14 mg protein (a)] in 1.0 ml of buffer and were incubated for 20 (b-e) or 120 min (a) at  $30^\circ\text{C}$ . Buffers were sodium citrate/phosphate for pH 5-7, sodium phosphate for pH 7-8, or glycine/NaOH/sodium phosphate for pH 8-10. Ordinate: pmol/min/mg protein.

#### pH optima of enzymes and pH of midgut cytosol

The pH optimum of ecdysone oxidase and  $3\alpha$ -reductase (NADH) was 7.5, the pH optimum of both  $3\beta$ -reductases was 6.5, and the  $3\alpha$ -reductase (NADPH) showed dual pH optima below 5.3 and above 9.7 (Fig. 2). The pH of the midgut cytosol was 7.9.

#### Kinetic parameters

For further characterization of the 3-oxoeecdysteroid 3-reductases the apparent kinetic parameters relative to the nicotinamide adenine dinucleotide co-

substrates were determined.  $3\alpha$ -Reductase (NADH) and  $3\beta$ -reductase (NADPH) showed Michaelis-Menten type relationships between cosubstrate concentrations and rates of reaction (Figs 3 and 4), but the other two reductases did not (Fig. 3). The kinetic parameters were  $K_m = 80.8 \pm 10.8 \mu\text{M}$  and  $V_{max} = 0.58 \pm 0.30 \text{ nmol/min/mg protein}$  for  $3\alpha$ -reductase (NADH), and  $K_m = 19.3 \pm 2.5 \mu\text{M}$  and  $V_{max} = 4.39 \pm 0.40 \text{ nmol/min/mg protein}$  for  $3\beta$ -reductase (NADPH) (means  $\pm$  SD, duplicate determinations from each of two separate experiments utilizing two different G-25 sup preparations). The

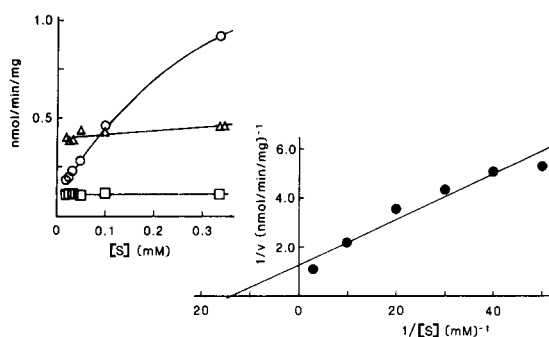


Fig. 3. Double reciprocal plot and/or velocity vs substrate-concentration plots for 3 $\alpha$ -reductase (NADH) (●, ○), 3 $\alpha$ -reductase (NADPH) (△), and 3 $\beta$ -reductase (NADH) (□). Incubation mixtures contained 25  $\mu$ M 3D, 0.55 mg protein/ml, NAD<sup>+</sup>, and regenerating system; or 12.5  $\mu$ M 3D, 0.085 mg protein/ml, NADP<sup>+</sup>, and regenerating system in buffer B and were incubated for 10 min at 30°C. Rates shown as nmol/min/mg protein.

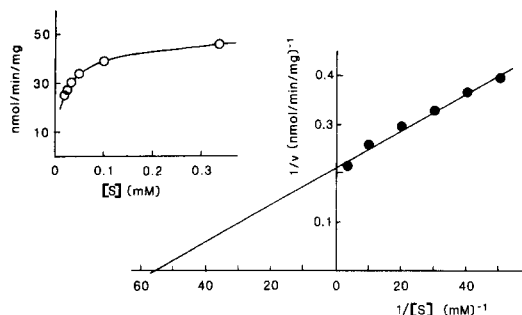


Fig. 4. Velocity vs substrate-concentration and double reciprocal plot for 3 $\beta$ -reductase (NADPH). Incubation mixtures contained 12.5  $\mu$ M 3D, 0.085 mg protein/ml, NADP<sup>+</sup>, and regenerating system in buffer B, and were incubated for 10 min at 30°C. Rates shown as nmol/min/mg protein.

data for 3 $\alpha$ -reductase (NADPH) and 3 $\beta$ -reductase (NADH) shown in Fig. 3, insert, might suggest that the cosubstrate concentrations used were too high and, therefore, the incubations failed to show a dependence of the reaction rates on the cosubstrate concentrations. However, other experiments conducted with lower cosubstrate concentrations (5–25  $\mu$ M NADH or NADPH) also failed to show a Michaelis–Menten type relationship.

#### Effects of NAD<sup>+</sup> and NADP<sup>+</sup> on 3-oxoecdysteroid 3-reductases

Many oxidoreductase-catalyzed reactions are reversible and the rates can be influenced by the concentrations (concentration ratios) of the substrates and/or products. We, therefore, determined the effects of NAD<sup>+</sup> and NADP<sup>+</sup> (at 8.3 times the concentration of NADH or NADPH) on the two major 3-oxoecdysteroid 3-reductases (Table 1). 3 $\alpha$ -Reductase (NADH) showed a 26% inhibition by NAD<sup>+</sup>, and a 100% inhibition by NADP<sup>+</sup>, and 3 $\beta$ -reductase (NADPH) showed 6% inhibition by NAD<sup>+</sup>, and 96% inhibition by NADP<sup>+</sup>. These results suggested that NADP<sup>+</sup> competes very effectively for the NADH binding site of the 3 $\alpha$ -reductase and for the NADPH binding site of the 3 $\beta$ -reductase. NAD<sup>+</sup>, on the other hand, seemed to exert moderate competition for the NADH binding site of the 3 $\alpha$ -reductase, but only marginal competition for the NADPH binding site of the 3 $\beta$ -reductase.

We also tested the reversibility of the 3-oxoecdysteroid reductions by incubating G-25 sup (2.2–3.5 mg protein/ml) with E or E' (25–50  $\mu$ M) and NAD<sup>+</sup> or NADP<sup>+</sup> (each at 0.6 and 2.5 mM) for up to 120 min. Although initial experiments indicated that 3D was formed in small amounts in such incubations (Weirich *et al.*, 1990b), these results could not be verified in later experiments, and the question of the reversibility of the 3-oxoecdysteroid reductions remains unresolved.

#### Effects of NaCl on 3-oxoecdysteroid 3-reductases

Sodium chloride had a very strong effect on the 3-oxoecdysteroid 3-reductases, especially the 3 $\alpha$ -reductase(s) (Table 2). The NADH-dependent reduction of 3D was 50% inhibited in the presence of 0.2 M NaCl, but the same salt concentration elicited a substantial NADPH-dependent 3 $\alpha$ -reduction that was undetectable in the absence of NaCl. The NaCl apparently changed the cosubstrate specificity or preference of the 3 $\alpha$ -reductase and at the same time enhanced the total activity. At 2.0 M, NaCl completely inhibited all 3 $\alpha$ -reductase activity. These results have been confirmed by experiments with a partially purified 3 $\alpha$ -reductase preparation (unpublished results). 3 $\beta$ -Reductase (NADPH) was 17% inhibited by 0.2 M NaCl, and 2.0 M NaCl caused a 94% inhibition.

Table 1. Effects of oxidized nicotinamide adenine dinucleotides on the two major 3-oxoecdysteroid 3-reductases

| Additions                              | 3 $\alpha$<br>(% of control) | 3 $\beta$<br>(% of control) |
|--|------------------------------|-----------------------------|
| 0.6 mM NADH                            | 100                          |                             |
| 0.6 mM NADH, 5.0 mM NAD <sup>+</sup>   | 74.2 $\pm$ 1.1               |                             |
| 0.6 mM NADH, 5.0 mM NADP <sup>+</sup>  | 0                            |                             |
| 0.6 mM NADPH                           |                              | 100                         |
| 0.6 mM NADPH, 5.0 mM NAD <sup>+</sup>  |                              | 93.9 $\pm$ 1.8              |
| 0.6 mM NADPH, 5.0 mM NADP <sup>+</sup> |                              | 3.7 $\pm$ 5.2               |

Incubation mixtures contained 25  $\mu$ M 3D; 0.30 mg protein/ml of buffer B (pH 7.5), with NADH as cosubstrate; or 0.90 mg protein/ml of buffer C (pH 6.5), with NADPH as cosubstrate; and were incubated for 10 min at 30°C. Means  $\pm$  SD of duplicate determinations of two separate experiments (two different enzyme preparations). The activities obtained in the control incubations of the two experiments (= 100%) were 1.12  $\pm$  0.07 and 1.43  $\pm$  0.06 nmol/min/mg protein for 3 $\alpha$ -reductase; 1.77  $\pm$  0.09 and 5.19  $\pm$  0.28 nmol/min/mg protein for 3 $\beta$ -reductase.

Table 2. Effect of NaCl on 3-oxoecdysteroid 3-reductases

| Additions                | 3 $\alpha$<br>(nmol/min/mg protein) | 3 $\beta$<br>(nmol/min/mg protein) |
|--------------------------|-------------------------------------|------------------------------------|
| 0.6 mM NADH              | 0.81 $\pm$ 0.06                     | 0                                  |
| 0.6 mM NADH, 0.2 M NaCl  | 0.40 $\pm$ 0.01                     | 0                                  |
| 0.6 mM NADH, 2.0 M NaCl  | 0                                   | 0                                  |
| 0.6 mM NADPH             | 0                                   | 6.68 $\pm$ 0.09                    |
| 0.6 mM NADPH, 0.2 M NaCl | 2.46 $\pm$ 0.14                     | 5.54 $\pm$ 0.16                    |
| 0.6 mM NADPH, 2.0 M NaCl | 0                                   | 0.41 $\pm$ 0.06                    |

Incubation mixtures contained 25  $\mu$ M 3D; 0.19 mg protein/ml of buffer B (pH 7.5), with NADH as cosubstrate; or 0.11 mg protein/ml of buffer C (pH 6.5), with NADPH as cosubstrate; and were incubated for 10 min at 30°C. Means  $\pm$  SD of duplicate determinations.

## DISCUSSION

In a previous communication we reported the apparent kinetic parameters of the five enzyme activities involved in ecdysteroid 3-epimerization in *M. sexta* midgut relative to their ecdysteroid substrates (Weirich *et al.*, 1990a). The evaluation of these data led to the conclusion that 3-epimerization of 3 $\beta$ -hydroxyecdysteroids is accomplished by the combined action of ecdysone oxidase and 3 $\alpha$ -reductase (NADH), with ecdysone oxidase being the rate-limiting enzyme and the 3 $\alpha$ -reductase converting most of the 3-oxo intermediate to the corresponding 3 $\alpha$ -hydroxyecdysteroid. This conclusion implied the assumption that the midgut cells contain predominantly NADH and very little NADPH. NADH/NADPH ratios in insect midguts have not been determined, but insect flight muscles have shown very high NADH/NADPH ratios (Candy, 1985). A low NADH/NADPH ratio (i.e. substantial NADPH content) in midgut cells would favor the 3 $\beta$ -reduction of the 3-oxo intermediate to the biologically active 3 $\beta$ -hydroxyecdysteroid, and *in situ* changes in the NADH/NADPH ratio could affect the balance between 3 $\alpha$ - and 3 $\beta$ -reduction.

The pH optimum of 7.5 for ecdysone oxidase and 3 $\alpha$ -reductase (NADH) was in good agreement with the pH optimum of 7.3 previously obtained for the complete E to E' conversion (Mayer *et al.*, 1979), and was close to the pH (7.9) of the midgut cytosol. Among the five enzymes (activities) analyzed, ecdysone oxidase and 3 $\alpha$ -reductase (NADH) seemed to be functionally best adapted to their physiological environment. This observation supports the conclusion that these two enzymes are dedicated to the 3-epimerization in midgut cells.

In the present study we undertook a kinetic analysis of the four 3-oxoecdysteroid 3-reductases by

varying the concentrations of the nicotinamide adenine dinucleotide cosubstrates. These experiments showed that only the two major reductases, 3 $\alpha$ -reductase (NADH) and 3 $\beta$ -reductase (NADPH), followed Michaelis-Menten type kinetics, whereas the other two reductases did not show significant changes in the reaction rates over a wide range of cosubstrate concentrations.

The Michaelis constants showed that the 3 $\alpha$ -reductase required a four times higher cosubstrate concentration (80.8 vs 19.3  $\mu$ M) for half maximal activity than the 3 $\beta$ -reductase (Table 3). The  $V_{\max}$  for 3 $\beta$ -reductase (NADPH) (4.39  $\pm$  0.40 pmol/min/mg protein) was determined at a 3D concentration of 12.5  $\mu$ M ( $K_m$  for 3D = 17.7  $\pm$  10.3  $\mu$ M; Weirich *et al.*, 1990a) and was, as expected, about one half of the  $V_{\max}$  previously obtained (10.7  $\pm$  3.3 pmol/min/mg protein) with varying 3D concentrations and a constant NADPH concentration of 600  $\mu$ M (30  $\times$   $K_m$ ). The  $V_{\max}$  for 3 $\alpha$ -reductase (NADH) (0.58  $\pm$  0.30 pmol/min/mg protein), similarly determined at a 3D concentration (25  $\mu$ M) equivalent to the  $K_m$  (30.7  $\pm$  16.2  $\mu$ M), was substantially lower than that found previously with varying 3D concentrations and a constant NADH concentration of 600  $\mu$ M (7.5  $\times$   $K_m$ ) (4.06  $\pm$  2.20 pmol/min/mg protein). This difference is probably due to the variations in this enzyme activity between different midgut cytosol preparations (Weirich *et al.*, 1990a).

Our results have shown a moderate inhibitory effect of NAD<sup>+</sup> and a strong inhibitory effect of NADP<sup>+</sup> on the two major 3-oxoecdysteroid 3-reductases. It is interesting to note that NADP<sup>+</sup> strongly affects 3 $\alpha$ -reductase (NADH) whereas NAD<sup>+</sup> had only a marginal effect on 3 $\beta$ -reductase (NADPH). These inhibitory effects may be caused by competition of the oxidized cosubstrates for the

Table 3. Apparent kinetic parameters of 3 $\alpha$ -reductase (NADH) and 3 $\beta$ -reductase (NADPH)\*

|                                 | 3D†                 |                     | NADH†               |                     | NADPH†              |                     | $V_{\max}$<br>(nmol/min/mg protein)                   |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---|
|                                 | conc.<br>( $\mu$ M) | $K_m$<br>( $\mu$ M) | conc.<br>( $\mu$ M) | $K_m$<br>( $\mu$ M) | conc.<br>( $\mu$ M) | $K_m$<br>( $\mu$ M) |   |
| 3 $\alpha$ -Reductase<br>(NADH) | 25.0                | 30.7 $\pm$ 16.2‡    | 600                 | 80.8 $\pm$ 10.8     | 600                 |                     | 4.06 $\pm$ 2.20‡                                      |
| 3 $\beta$ -Reductase<br>(NADPH) | 12.5                | 17.7 $\pm$ 10.3‡    |                     |                     |                     | 19.3 $\pm$ 2.5      | 0.58 $\pm$ 0.30<br>10.7 $\pm$ 3.3‡<br>4.39 $\pm$ 0.40 |

\*Means  $\pm$  SD.

†Substrate or cosubstrate concentrations are shown only for experiments in which they were kept constant. Otherwise the  $K_m$  resulting from the kinetic analysis are shown.

‡From Weirich *et al.* (1990a).

binding sites for the reduced cosubstrates on the reductases (product inhibition).

Previously we tested the potential of E for product inhibition of the  $3\beta$ -reduction of 3D (Weirich *et al.*, 1990a). 3D was either infused into incubation mixtures or formed from E by the action of ecdysone oxidase at a comparable rate. Although these incubations had a more than 1000-fold excess of E over 3D (E at  $50\text{ }\mu\text{M}$ , 3D in the nM range), no product inhibition was observed. E' has been shown to exert weak (product?) inhibition on the complete reaction sequence from E to E' (Mayer *et al.*, 1979), but it is not known whether E' inhibited ecdysone oxidase or  $3\alpha$ -reductase.

The apparent effectiveness of  $\text{NADP}^+$  for product inhibition of  $3\beta$ -reductase at an  $\text{NADP}^+/\text{NADPH}$  ratio of 8.3 and the failure of E to cause product inhibition at a E/3D ratio of  $>1000$  suggests that E does not compete with 3D for binding to free reductase, but  $\text{NADP}^+$  does compete with NADPH. In other words, the enzyme-cosubstrate complex formation probably precedes the binding of the ecdysteroid substrate to the enzyme-cosubstrate complex. Thus, our data are compatible with the assumption of an ordered bi bi mechanism with the cosubstrate binding to the free enzyme, and the ecdysteroid binding to the enzyme-cosubstrate complex. This has been shown to be the mechanism for  $3\alpha$ -hydroxysteroid dehydrogenase from mouse liver cytosol (Hara *et al.*, 1988), and  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase/3-keto- $\Delta^5$ -steroid isomerase from bovine adrenal microsomes (Brandt and Levy, 1989). Future work will have to prove whether it is indeed the mechanism for the action of corresponding *M. sexta* midgut cytosol enzymes as well.

Incubations of G-25 sup with E or E' and  $\text{NAD}^+$  or  $\text{NADP}^+$  failed to produce significant amounts of 3D. Thus, the 3-oxoecdysteroid 3-reductase reactions were not reversible under our experimental conditions, although the inhibition experiments indicated that  $\text{NAD}^+$  and  $\text{NADP}^+$  bind to the enzymes. In contrast to these findings, reactions catalyzed by mammalian  $3\alpha$ -hydroxysteroid dehydrogenases have been shown to be reversible (Penning *et al.*, 1984; Hara *et al.*, 1988).

Potassium ions (up to  $100\text{ }\mu\text{M}$ ) had little effect on the 3-epimerization when tested with a partially purified enzyme preparation (Mayer *et al.*, 1979). In our present study sodium chloride (at  $0.2\text{ M}$ ) showed a very profound effect on one (some) of the enzymes, 3-oxoecdysteroid  $3\alpha$ -reductase(s). It inhibited the activity with NADH as cosubstrate by about 50% and greatly increased the activity with NADPH as cosubstrate (from undetectable to 3-times the initial activity with NADH). The enzyme protein(s) seemed to have undergone a structural change under the influence of the NaCl that favored binding of NADPH over the binding of NADH and at the same time enhanced the total enzyme activity.  $3\beta$ -Reductase activity, on the other hand, was only moderately inhibited at  $0.2\text{ M}$  NaCl, and the cosubstrate preference was not affected. These data show that in addition to the NADH/NADPH ratio and the  $\text{NAD(P)}^+/\text{NAD(P)H}$  ratio, the concentrations of NaCl and perhaps those of other salts (ions) can change the balance between enzymatically

produced  $3\beta$ -hydroxyecdysteroids and  $3\alpha$ -hydroxyecdysteroids.

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